Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration

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Background: In the trunk of avian embryos, neural crest migration through the somites is segmental, with neural crest cells entering the rostral half of each somitic sclerotome but avoiding the caudal half. Little is known about the molecular nature of the cues – intrinsic to the somites – that are responsible for this segmental migration of neural crest cells.

Results: We demonstrate that Eph-related receptor tyrosine kinases and their ligands are essential for the segmental migration of avian trunk neural crest cells through the somites. EphB3 localizes to the rostral half-sclerotome, including the neural crest, and the ligand ephrin-B1 has a complementary pattern of expression in the caudal half-sclerotome. To test the functional significance of this striking asymmetry, soluble ligand ephrin-B1 was added to interfere with receptor function in either whole trunk explants or neural crest cells cultured on alternating stripes of ephrin-B1 *versus* fibronection. Neural crest cells *in vitro* avoided migrating on lanes of immobilized ephrin-B1; the addition of soluble ephrin-B1 blocked this inhibition. Similarly, in whole trunk explants, the metameric pattern of neural crest migration was disrupted by addition of soluble ephrin-B1, allowing entry of neural crest cells into caudal portions of the sclerotome.

Conclusions: Both *in vivo* and *in vitro*, the addition of soluble ephrin-B1 results in a loss of the metameric migratory pattern and a disorganization of neural crest cell movement. These results demonstrate that Eph-family receptor tyrosine kinases and their transmembrane ligands are involved in interactions between neural crest and sclerotomal cells, mediating an inhibitory activity necessary to constrain neural precursors to specific territories in the developing nervous system.

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Background

During embryonic development, neural crest cells migrate along stereotypic pathways to their target destinations, where they differentiate into much of the peripheral nervous system, including sensory and sympathetic ganglia [1,2]. In the trunk of the avian embryo, neural crest migration through the somite is segmental, with neural crest cells entering the rostral half of each somitic sclerotome but avoiding the caudal half [3,4]. Previous studies have shown that cues intrinsic to the somites are responsible for this segmental migration and that similar mechanisms may influence the guidance of motor and sensory axons [5–7]. Inhibitory molecules localized to caudal sclerotomes and/or attractive molecules present in rostral sclerotomes could contribute to segmental patterning. A number of candidate molecules have the expected spatiotemporal localization: for example, chondroitin sulfate proteoglycans [8–10], T-cadherin [11] and peanut agglutinin (PNA)-binding molecules [8] are all expressed in the caudal half-sclerotome, whereas butyrylcholinesterase is expressed in the rostral half-sclerotome [12]. However, functional experiments are required to elucidate the role, if any, of these molecules in cell migration. We recently developed an explant preparation that allows direct visualization of neural crest migration in normal living tissue and offers accessibility to perturbing reagents [13,14]. Here, we report functional studies demonstrating a role for Eph receptor–ligand interactions in neural crest migration.

Several members of the Eph family of receptor tyrosine kinases and their ligands display intriguing patterns of expression in the developing nervous system, suggesting that they may play a role in early neural patterning [15–20]. On the basis of their binding specificities, the Eph receptors can be divided into two major subclasses: EphB-related receptors, which interact primarily with a

transmembrane subclass of ligands, and EphA-related receptors, which interact primarily with a glycosylphosphatidylinositol-linked subclass of ligands [15,18]. Eph receptor–ligand interactions have been implicated in axonal patterning events, such as the topographic organization of the retinotectal projection and axonal fasciculation [21–25]. Members of this family also are expressed in the developing forebrain and hindbrain [16,20,26–28] and appear to play a role in their regional organization [28–30]. The potential role of these molecules in the patterning of neural precursors in the developing peripheral nervous system remains largely unexplored. (In this manuscript, we refer to the Eph family receptors and ligands using a new nomenclature that has been agreed upon by the Eph Nomenclature Committee [31].)

Results

The EphB3 receptor is expressed in the neural crest and rostral sclerotome

To detect receptors of the EphB subclass during trunk neural crest migration, a fusion protein comprising a representative transmembrane ligand, ephrin-B1, and the Fc portion of human IgG1 (ephrin-B1-Fc) was used to stain whole chick embryos [18,32]. Ligand–receptor interactions among members of a subclass are known to be promiscuous, and the ligand ephrin-B1-Fc has the potential to interact with receptors EphB1 (Elk/Cek6), EphB2 (Nuk/Cek5/Qek5) and EphB3 (Hek2/Sek4/Cek10), but not EphB4 (Htk/MDK2/Myk1) [18,19].

Receptors recognized by ephrin-B1-Fc appeared first in the rostral portion of mature somites at the 15-somite stage (stage 12 [33]), coincident with the onset of neural crest migration (data not shown); this pattern persisted and became more distinct by the 25-somite stage (stage 15; Figure 1a) through the 35-somite stage (stage 19). Longitudinal sections revealed that the receptors were associated with the surface of cells in the rostral half-sclerotome (Figure 1b). Identical results were observed in chick (Figure 1) and quail (data not shown) embryos.

To identify the specific Eph receptors that may account for the staining associated with neural crest migratory pathways, we carried out *in situ* hybridizations with quail-specific probes for two members of the EphB subclass, *EphB3* [34], and *EphB2* [24,35]. At the forelimb level of the 25-somite embryo, *EphB3* transcripts localized within cells in the rostral half-sclerotome during neural crest migration (Figure 1c). In contrast, no transcripts were apparent in the 4–5 most recently formed epithelial somites, prior to both the time of sclerotome formation and the entry of neural crest cells. Double-labeling with anti-HNK-1 antibody, a neural crest marker, showed that all neural crest cells expressed *EphB3* mRNA (Figure 1d–f). Other *EphB3*-positive cells in the rostral half-sclerotome and dermomyotome were HNK-1-negative, and even extended into the perinotochordal space, which is devoid of neural crest cells. These labelling patterns indicate that cells within the rostral half-somite other than neural crest cells also express *EphB3*. In contrast to *EphB3*, *EphB2* was expressed weakly in the rostral sclerotome only at later stages of neural crest migration (data not shown). These results suggest that migrating neural crest cells express EphB3, consistent with their ability to be recognized by ephrin-B1-Fc.

The ephrin-B1 ligand is distributed in the caudal sclerotome

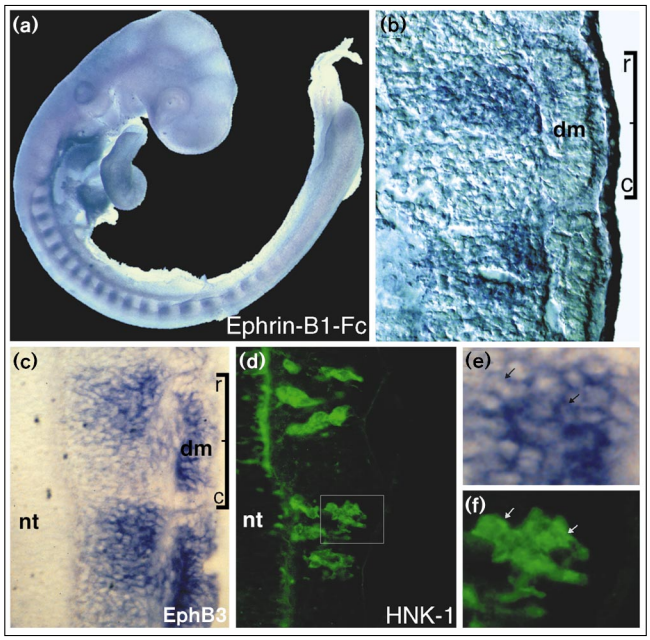
To investigate the distribution of transmembrane ligands during the process of trunk neural crest migration, a representative receptor-Fc fusion protein (EphB2-Fc) was used to label whole chick embryos. EphB2-Fc has the potential to interact with Eph family transmembrane ligands, including ephrin-B1, ephrin-B2 and ephrin-B3 [18,36]. At the 10-somite stage, ligands recognized by EphB2-Fc were expressed highly in the caudal portion of all somites, including immature epithelial somites (prior to neural crest migration). During active neural crest migration, EphB2-Fc staining persisted in the caudal half of each somite (Figure 2a; 25-somite stage). Longitudinal sections showed that ligands were associated with the surfaces of the caudal half-sclerotome and some dermomyotome cells (Figure 2b). Throughout neurulation, staining was present within the open neural plate (data not shown) and in the recently closed neural tube (Figure 2a).

To identify the ligands bound by EphB2-Fc in the caudal half-sclerotome, *in situ* hybridization was carried out in whole chick embryos with probes for *ephrin-B1* and *ephrin-B2* mRNA [37]. In 25-somite embryos, *ephrin-B1* mRNA was observed in a segmental pattern in the caudal halves of somites along the body axis. Longitudinal sections confirmed that *ephrin-B1* transcripts were expressed by caudal half-sclerotome cells (Figure 2c), and were absent from the rostral half-sclerotome through which neural crest cells migrate (Figure 2d). *Ephrin-B2* was not expressed in the somite, but was localized to endothelial cells positioned at the intersomitic boundary (Figure 2e). The chick homolog of *ephrin-B3* has not been isolated and so its pattern of expression during trunk neural crest migration was not investigated.

Soluble ephrin-B1 disrupts the segmental pattern of neural crest migration in whole trunk explants

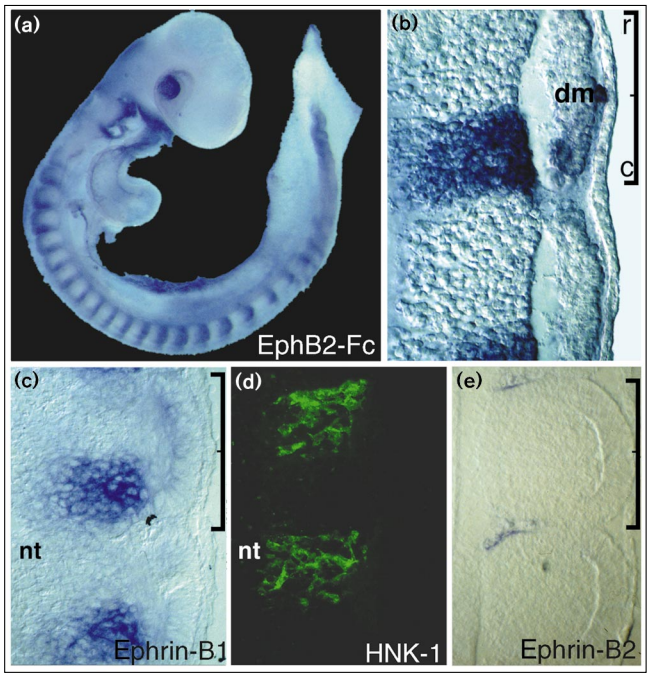
The reciprocal distribution of receptors and ligands suggests that they may play a role in conferring the segmental pattern of neural crest migration. Specifically, neural crest cells expressing EphB3 may interact with caudal sclerotome cells expressing ephrin-B1, mediating an inhibitory event that prohibits entry of neural crest into this caudal domain. To test this hypothesis, soluble ephrin-B1-Fc was added to whole trunk explants [13] to disrupt receptor–ligand interactions. The subsequent distribution

Figure 1



Staining of 25-somite chick embryos with ephrin-B1-Fc and hybridization with an *EphB3* cDNA probe reveals that Eph receptors are distributed on cells in the rostral half of the somitic sclerotome, including neural crest. **(a)** In a whole-mount preparation, ephrin-B1-Fc staining, visualized with anti-human Fc antibody coupled to alkaline phosphatase, is distributed in a segmental fashion in the developing somites. **(b)** A longitudinal section shows ephrin-B1-Fc staining localized primarily to rostral sclerotome; the brackets indicate the rostrocaudal extent of the somite, with the tick-mark representing the intrasomitic border. **(c)** A longitudinal section of another 25-somite embryo after whole-mount *in situ* hybridization shows that *EphB3* transcripts are present throughout the rostral sclerotome. **(d)** The section in (c) was stained with anti-HNK-1 antibody to mark migrating neural crest cells within the rostral sclerotome. **(e,f)** A higher magnification view of the boxed region in (d) shows that all HNK-1-positive neural crest cells in the rostral sclerotome express *EphB3* mRNA; small arrows mark two examples of double-labeled cells. Other rostral sclerotomal, dermomyotomal and ectodermal cells are recognized by ephrin-B1-Fc and EphB3 cDNA, but not HNK-1 antibody. Abbreviations: r, rostral; c, caudal; nt, neural tube; dm, dermomyotome.

Figure 2

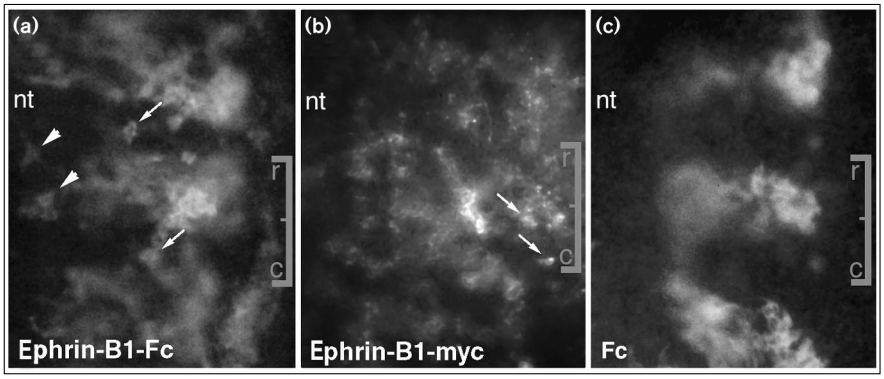


Staining of 25-somite chick embryos with EphB2-Fc and hybridization with an *ephrin-B1* cDNA probe reveals that transmembrane ligands are distributed on cells in the caudal sclerotome. **(a)** In a whole-mount preparation, EphB2-Fc staining, visualized with anti-human Fc antibody coupled to alkaline phosphatase, is found in the caudal halves of somites. **(b)** A longitudinal section through the trunk reveals EphB2-Fc staining in caudal sclerotome as well as the dermomyotome. **(c)** A longitudinal section through an embryo after *in situ* hybridization with an *ephrin-B1* cDNA probe reveals that *ephrin-B1* transcripts are present in caudal sclerotomal cells. **(d)** The same section as (c) after staining with anti-HNK-1 antibody to recognize migrating neural crest cells reveals that *ephrin-B1* is expressed in a pattern complementary to the distribution of neural crest cells. **(e)** A longitudinal section of an embryo after hybridization with an *ephrin-B2* probe reveals that these transcripts localize to endothelial cells at intersomitic borders, but not sclerotome. Brackets indicate the rostrocaudal extent of a single somite, with the midline representing the intrasomitic border. Abbreviations: r, rostral; c, caudal; nt, neural tube; dm, dermomyotome.

of migrating neural crest cells was assayed by anti-HNK-1 antibody labeling or by pre-labeling the neural tube with the lipophilic dye DiI [38]. In the presence of ephrin-B1-Fc (1 μg/ml or 10 μg/ml), the normally segmental migratory pattern of neural crest cells was disrupted: labeled cells were observed in both rostral and caudal portions of the sclerotome (Figure 3a; Table 1; $n = 42$), as confirmed by sectioning the explants (data not shown). In the presence of 10 μg/ml monomeric ephrin-B1-myc — used to test whether the disruption resulted from the dimeric nature of ephrin-B1-Fc — neural crest cells were again observed in both halves of the somite (Figure 3b; $n = 16$). In contrast, treatment with soluble Fc (Figure 3c; 10 μg/ml; $n = 20$), COS cell supernatant (concentrated similarly to ephrin-B1-myc; $n = 12$), or soluble EphB2-Fc (10–50 μg/ml; $n = 25$; data not shown) did not alter the segmental pattern of neural crest migration.

To quantitate these effects on neural crest patterning, we counted the numbers of cells in the rostral and caudal halves of somites treated with ephrin-B1-Fc, ephrin-B1-myc or Fc ($n = 16$ somites in 8 explants per condition; Table 1). In Fc-treated somites, 2% (8/488) of the neural crest cells entered the caudal sclerotome. In contrast, 33% (296/896) of neural crest in ephrin-B1-Fc-treated somites and 47% (486/1035) of neural crest in ephrin-B1-myc-treated somites entered the caudal sclerotome (Table 1). These results indicate that binding the EphB3 receptor with excess, soluble ephrin-B1 protein allows neural crest cells to enter the caudal sclerotome domain.

Figure 3



Soluble ephrin-B1 disrupts the segmental pattern of neural crest migration. Ephrin-B1-Fc (a), ephrin-B1-myc (b) or Fc alone (c) were added to trunk explants and static patterns of neural crest migration were visualized by anti-HNK-1 antibody staining. **(a,b)** Neural crest cells enter both rostral and caudal-half somites in the presence of either dimeric ephrin-B1-Fc (a) or monomeric ephrin-B1-myc (b). **(c)** In Fc-treated control explants, neural crest migrate in their typical segmental pattern through the rostral half of the somite. Effects were observed with ephrin-B1-Fc at 1 μg/ml or 10 μg/ml; treatment with 250 ng/ml and 500 ng/ml had little or no effect. Arrowheads indicate cells over the dorsal aspect of the neural tube. Small arrows mark a few examples of neural crest cells migrating within the caudal portion of the somite. Brackets indicate the rostrocaudal extent of a single somite, with the midline representing the intrasomitic border. Abbreviations: r, rostral; c, caudal; nt, neural tube.

Time-lapse analysis of neural crest migration in the presence of ephrin-B1

To define more clearly the defects in cell migration caused by the presence of ephrin-B1-Fc, DiI-labeled neural crest cells were followed in explants using time-lapse videomicroscopy. In control explants [13], the neural crest cells entered only the rostral half-sclerotome. Exposure to soluble ephrin-B1-Fc resulted in neural crest cells emigrating directly from the neural tube into both rostral and caudal somitic halves (Figure 4).

The trajectories and rates of migration for individual cells in trunk explants treated with ephrin-B1-Fc, EphB2-Fc or Fc were acquired from the time-lapse recordings with an automated cell-tracking program (S. Speicher and J. Solomon, unpublished). In ephrin-B1-Fc-treated explants, the trajectories of cells in both the rostral and caudal somite halves appeared more erratic and disorganized than those in control cultures (Figure 4). Surprisingly, the ephrin-B1-Fc-treated cells often moved backwards from the somite to the neural tube. Quantitative measurements of cell migration showed that the mean rates of migration were not significantly different between ephrin-B1-Fc-treated cells (10.8 ± 3.1 μm/h; $n = 53$), untreated cells (14.9 ± 3.0 μm/h; $n = 12$), EphB2-Fc-treated cells (13.7 ± 3.8 μm/h; $n = 10$) or Fc-treated cells (17.6 ± 2.8 μm/h; $n = 14$), and were similar to those reported previously [13]. However, ephrin-B1-Fc treatment caused significant differences in the directionality of cell migration — defined as the displacement distance of a cell divided by its total trajectory length — compared with Fc treatment or EphB2-Fc treatment (Table 2). This was manifest in the disordered migratory trajectories of neural crest cells in ephrin-B1-Fc-treated explants, compared with the linear migratory trajectories observed in controls (compare Figure 4g,h). Interestingly, differences in directionality were noted even at concentrations of ephrin-B1-Fc (for example, 250 ng/ml) too low to disrupt segmental migration of neural crest cells. Analysis of the cell trajectories of ephrin-B1-Fc-treated cultures showed some of them crossing the intrasomitic border (7/40 ephrin-B1-Fc-treated cells). In contrast, such crossings were observed only rarely under control conditions (1/14 Fc-treated cells and 0/12 untreated cells). Thus, the bearing and directionality of neural crest migration, but not its rate, were altered by exogenous ephrin-B1-Fc.

Table 1

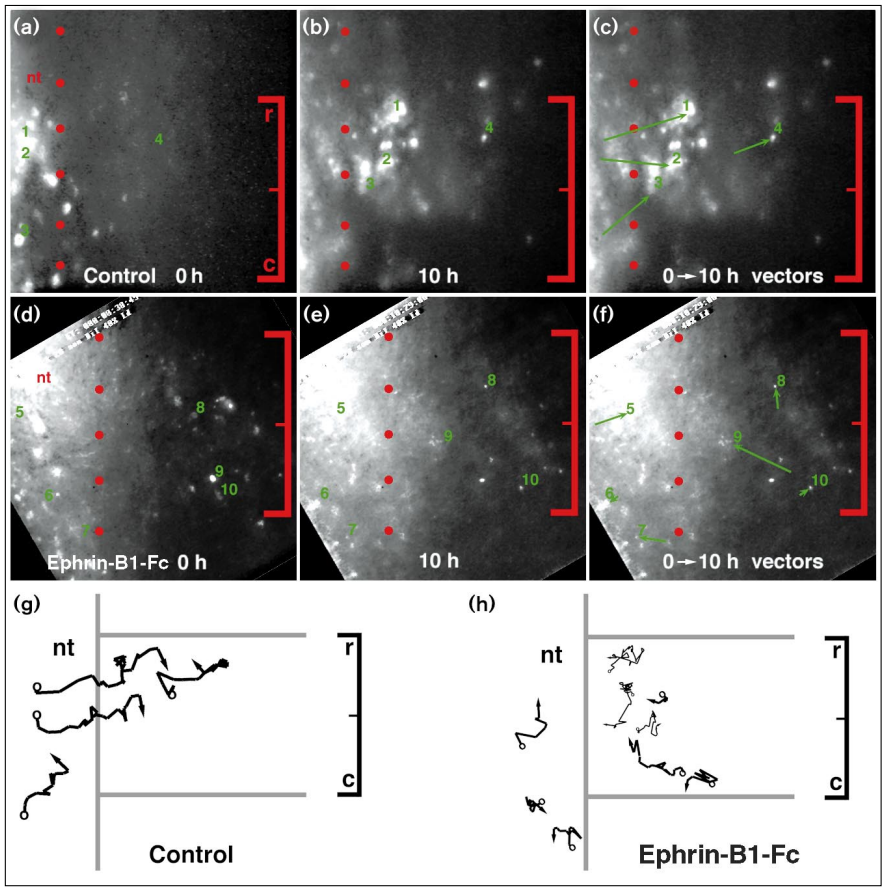
Effects of ephrin-B1 on the mean numbers of neural crest cells in rostral and caudal halves of the sclerotome.

Treatment	Number of somites/explants	Rostral	Caudal
Fc (control)	16/8	30.1 cells	0.5 cells
Ephrin-B1-Fc	16/8	35.8 cells	18.5 cells
Ephrin-B1-myc	16/8	34.3 cells	30.4 cells

The numbers of cells in the rostral and caudal halves of the sclerotome in whole explants treated with Fc alone, ephrin-B1-Fc or ephrin-B1-myc were counted in static images of whole trunk explants labeled with anti-HNK-1 antibody.

The disruptive effect of dimeric ephrin-B1-Fc does not appear to result from an alteration of the polarity of the somitic sclerotome. The distribution pattern of molecular markers characteristic of either the rostral or caudal half-sclerotome were normal in ephrin-B1-Fc-treated explants: heparan sulfate proteoglycans were present in their typical position in the rostral half-sclerotome; and PNA-binding glycoproteins and chondroitin sulfate proteoglycans were found in their characteristic location in the caudal half-sclerotome (data not shown).

Figure 4



Time-lapse analysis of individual cell migration in control and ephrin-B1-Fc-treated explants. DiI-labeled neural crest cells migrating in trunk explants were followed using time-lapse videomicroscopy with images acquired at 2 min intervals. This figure illustrates two representative frames taken at 0 and 10 h. The positions of four cells (cells 1–4) in control Fc-treated explants **(a–c)** and six cells (cells 5–10) in ephrin-B1-Fc-treated explants **(d–f)** were followed using a cell-tracking program. The green numbers overlie the initial (a,d) and later (b,c,e,f) positions of the cells. The arrows in (c,f) indicate the vectors between the two frames, with arrowheads marking the position of the cell after 10 h and the tail at the position of the cell at its initial time of observation. The length of the vector gives the total distance traveled in 10 h. The dotted red line represents the lateral edge of the neural tube (nt). The red bracket indicates the rostrocaudal extent of the somites, with the intrasomitic border in the midline; r, rostral; c, caudal. **(g,h)** Schematic of trajectories of neural crest cells in control Fc (g) and ephrin-B1-Fc-treated (h) explants. Vectors of individual cell movements were plotted at 30 min time intervals and joined to record the trajectories of individual neural crest cells. The cell migration vectors shown are representative of several time lapse sequences; cells tracked from different films are indicated by differing line thicknesses. Under control conditions, cell moved in an overall linear manner in the somites and were constrained to the rostral half of the sclerotome. In contrast, cell movement in ephrin-B1-Fc-treated cultures was tortuous, with cells located in both halves of the sclerotome moving in a random, disorganized fashion.

Ephrin-B1 inhibits neural crest migration *in vitro*

The trunk explant system cannot distinguish whether exogenous ephrin-B1 acts directly on neural crest cells or indirectly by binding to rostral sclerotome. We therefore examined the direct effects of exogenous ephrin-B1 protein on neural crest migration *in vitro*. Neural crest cells were plated on a patterned two-dimensional substrate composed of alternating stripes [39,40] of multimeric ephrin-B1-Fc plus fibronectin *versus* fibronectin alone. The behavior of these cells was compared with cells on control substrates composed of: alternating stripes of Fc plus fibronectin *versus* fibronectin alone; alternating stripes of fibronectin alone; or alternating stripes of anti-Fc antibody plus fibronectin *versus* fibronectin alone.

Cultured neural crest cells bound ephrin-B1-Fc, indicating that they maintained expression of receptors (Figure 5a,b). The cells migrated uniformly on all control substrates (Figure 5c), but individual cells avoided migrating on stripes containing ephrin-B1-Fc ($n = 31$) and instead migrated on stripes composed of fibronectin alone (Figure 5d). Time-lapse videomicroscopy of migrating neural crest on these striped substrates indicated that the cells displayed a typical avoidance response consisting of lamellipodial collapse and process retraction in the

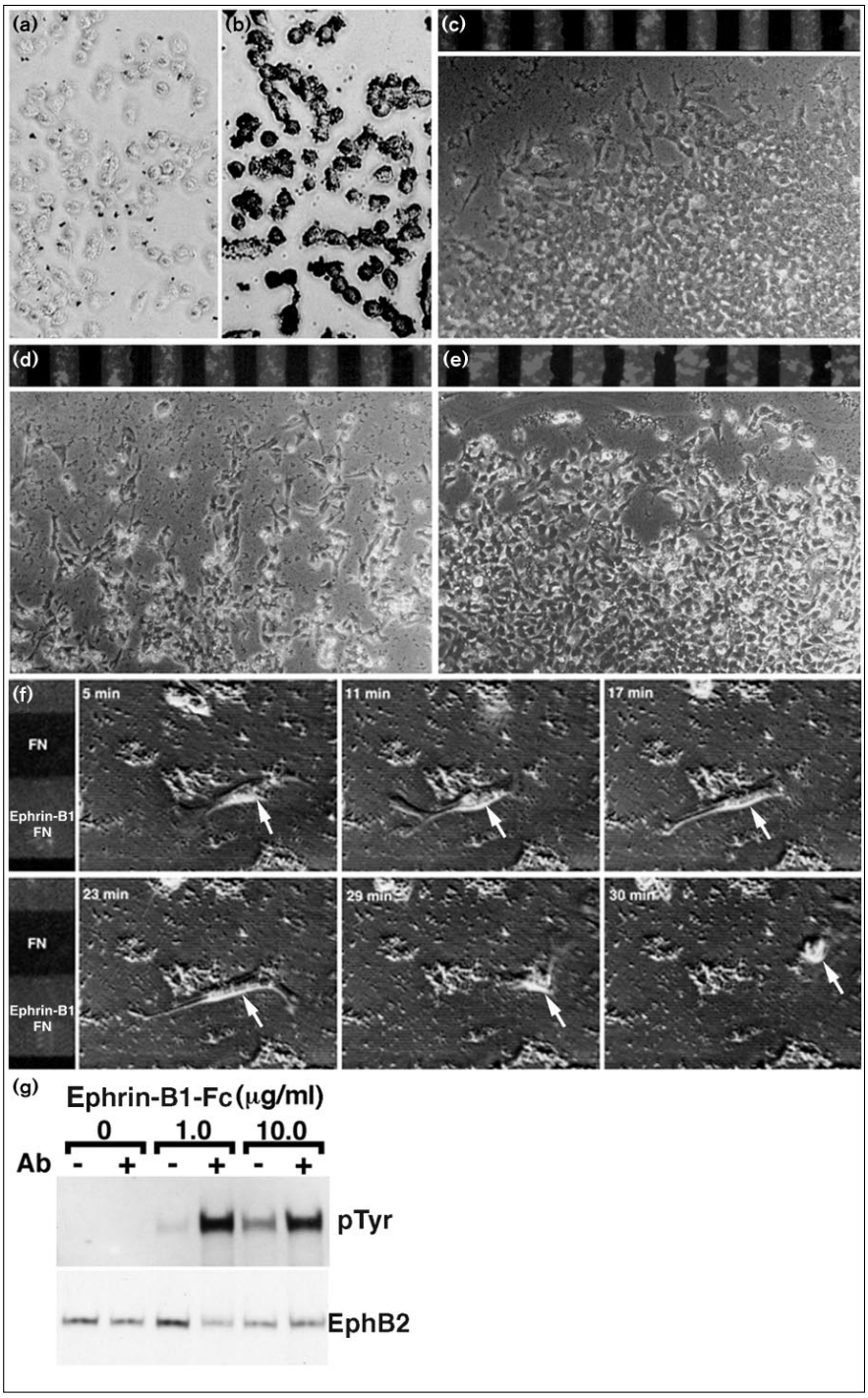
Table 2

The effects of ephrin-B1 and EphB2 on the directionality of neural crest migration.

Treatment	Concentration	Number of explants	Directionality
Fc (control)	10 μg/ml	14	0.37 ± 0.11
Ephrin-B1-Fc	250 ng/ml	7	0.20 ± 0.14*
Ephrin-B1-Fc	1 μg/ml	41	0.19 ± 0.07*
EphB2-Fc	10 μg/ml	10	0.39 ± 0.13

The directionality of neural crest cell migration, defined as the displacement distance divided by the net distance, was determined for explants treated with Fc alone, with ephrin-B1-Fc or with EphB2-Fc. Significantly different levels of directionality were noted between experimental and control conditions, even in low concentrations of ephrin-B1-Fc which caused no obvious defects in the pattern of migration; *$p < 0.001$.

Figure 5



Alternating lanes of ephrin-B1-Fc *in vitro* inhibit neural crest migration in a manner that is mitigated by the addition of soluble ephrin-B1-Fc. This avoidance by neural crest of ligand-Fc lanes involves process collapse/retraction. **(a,b)** Neural crest cells grown *in vitro* were labeled with Fc alone (a) or ephrin-B1-Fc (b) and visualized with anti-human Fc antibody coupled to alkaline phosphatase. (a) Control cultures displayed no staining. (b) Ephrin-B1-Fc bound specifically to neural crest cells, indicating that receptor expression was maintained *in vitro*. **(c)** When migrating neural crest cells where exposed to a substrate composed of alternating lanes of fibronectin *versus* Fc/fibronectin, they migrated uniformly on both substrates. **(d)** When migrating neural crest cells where exposed to a substrate composed of alternating lanes of fibronectin *versus* antibody-aggregated ephrin-B1-Fc/fibronectin, they migrated selectively on the fibronectin lanes, avoiding the lanes containing multimeric ephrin-B1-Fc. **(e)** When soluble ephrin-B1-Fc was added to the culture medium of neural crest migrating on alternating lanes of fibronectin *versus* antibody-aggregated ephrin-B1-Fc/fibronectin, they migrated equally well on all lanes. Analogous results were observed with monomer ephrin-B1-myc (data not shown). This demonstrates that soluble ephrin-B1 functions as a competitive inhibitor of multimeric ephrin-B1-Fc. **(f)** Time-lapse sequences acquired from 5–30 min of a migrating neural crest cell on striped substrates to examine ligand avoidance behavior (at 0 min, cell body was on the fibronectin (Fn) stripe). Neural crest cell enters ephrin-B1 lane and extends processes followed by process collapse and retraction. **(g)** Comparison of tyrosine phosphorylation of the EphB2 receptor in COS7 cells by dimeric and multimeric ephrin-B1-Fc. Ephrin-B1-Fc was added at concentrations ranging from 0–10 μg/ml; (–) indicates the addition of the dimeric form of ephrin-B1-Fc in the absence of added Fc antibody (Ab), and (+) indicates the addition of ephrin-B1-Fc in the presence of anti-Fc antibody. The levels of EphB2 protein are shown below each lane. At 1 and 10 μg/ml, dimeric ephrin-B1-Fc resulted in substantially lower levels of tyrosine phosphorylation than did multimeric ephrin-B1-Fc.

presence of ephrin-B1. The typical broad lamellipod on the leading process of a migrating neural crest cell underwent collapse and retraction after crossing into an ephrin-B1 stripe (Figure 5f). This avoidance behavior did not occur immediately upon contact with the border of ephrin-B1, but many minutes after neural crest entry and process extension on the ephrin-B1 stripe. In contrast, cells on control substrates did not demonstrate collapse behavior upon crossing stripes (data not shown).

To examine further the specificity of these effects, and to verify that soluble ephrin-B1 interferes with the normal receptor–ligand interactions, we studied the effects of adding soluble ephrin-B1 on the behavior of neural crest

cells on patterned ephrin-B1/fibronectin substrates. After the addition of soluble dimeric ephrin-B1-Fc (10 μg/ml; 1.7×10^{-7} M; $n = 10$), neural crest cells migrated equally well on all lanes (Figure 5e). Analogous results were obtained with monomeric ephrin-B1-myc (10 μg/ml; 3.3×10^{-7} M; $n = 3$). The similarity of the two effects argues that a major mechanism of action of soluble ligand is via competitive inhibition: ephrin-B1-Fc causes a small but significant receptor autophosphorylation (Figure 5g), whereas ephrin-B1-myc causes no signal transduction [32]. In contrast to ligand fusion proteins, soluble EphB2-Fc had no obvious effect on the patterned migration on ephrin-B1/fibronectin (data not shown). Our results demonstrate that lanes of ephrin-B1-Fc can directly inhibit neural crest migration and verify that soluble ephrin-B1 can function as a competitive antagonist of this inhibition.

Treatment with ephrin-B1 induces receptor phosphorylation

The phosphorylation status of endogenous EphB2 receptor expressed by COS7 cells was examined after the addition of 1 or 10 μg/ml of clustered or dimeric ephrin-B1-Fc. Even at 1 μg/ml, clustered ephrin-B1-Fc induced saturating levels of tyrosine phosphorylation of EphB2 (Figure 5g). In contrast, substantially lower levels of tyrosine phosphorylation were observed with 1 or 10 μg/ml of unclustered dimeric ephrin-B1-Fc. EphB2 tyrosine phosphorylation was not observed in untreated COS7 cells (Figure 5g) or in ephrin-B1-myc treated COS7 cells [32].

Discussion

In the present study, we have examined whether members of the Eph family of receptors and ligands participate in the segmental patterning of neural crest migration. Ligand-Fc and receptor-Fc fusion proteins were used to identify the distribution of their respective subclass of receptors and ligands [18]. Staining with ephrin-B1-Fc and *in situ* hybridization with receptor-specific probes showed the strong expression of EphB3 (Figure 1) and weak expression of EphB2 in the rostral half-sclerotome. The strong binding of ephrin-B1-Fc and expression of EphB3 by neural crest cells as they migrate is consistent with the previous observation that the ligand ephrin-B1 binds with high affinity to EphB3 [18,41]. The cognate ligands are present in the caudal half of each sclerotome, from which neural crest cells are normally absent, first appearing prior to neural crest migration (Figure 2). Using *in situ* hybridization with ligand-specific probes, we found that the ligand ephrin-B1, but not ephrin-B2, was expressed in the caudal half-sclerotome in a pattern consistent with EphB2-Fc labeling (Figure 2). In studies of axon guidance and fasciculation [42,43], an emerging theme is that interactions between Eph receptors and their ligands mediate repulsive signals that constrain growing axons to particular regions in the developing nervous system. The complementary expression patterns of the EphB3 receptor and the ephrin-B1 ligand in avian somites raises the intriguing possibility that Eph interactions constrain the migration of neural crest cells to the rostral sclerotome.

To test the functional significance of this complementary distribution of receptors and ligands, we examined neural crest migration both *in vivo* and *in vitro*. In a whole trunk explant system [13], the addition of soluble ephrin-B1 disrupted the patterned migration of neural crest cells in their native environment (Figure 3). Neural crest cells were observed within both rostral and caudal halves of each somitic sclerotome after exposure to ephrin-B1-myc or ephrin-B1-Fc, suggesting that the normal inhibitory action of the caudal half-sclerotome on neural crest cells had been blocked. A significant increase in the numbers of neural crest cells within the somites in the presence of ephrin-B1-myc or ephrin-B1-Fc compared with controls was noted. One possibility to account for this increase in cell number is that neural crest entered the somite prematurely in treated explants. Using an *in vitro* assay (alternating lanes of ephrin-B1/fibronectin), we determined that ephrin-B1 ligand can directly inhibit neural crest cells (Figure 5), causing collapse and retraction of cell processes, analogous to growth cone collapse behavior displayed by neurons in culture [21].

Of note, the repulsive activity of ephrin-B1 on migrating neural crest *in vitro* does not appear as an immediate consequence of cell contact with ligand, but instead develops over many minutes. The addition of soluble ephrin-B1 (either monomeric or dimeric) blocked this inhibition, such that neural crest cells migrated uniformly on all lanes. As monomeric ephrin-B1-myc fails to activate signal transduction [32], it functions solely as an antagonist; dimeric ephrin-B1-Fc activates low levels of receptor phosphorylation (Figure 5g) and may function as both an antagonist and a weak agonist. These results show that substrate-bound ephrin-B1 directly inhibits neural crest cell migration, and that soluble ephrin-B1 disrupts this inhibition. Thus, soluble ephrin-B1 acts as a competitive inhibitor of ligand binding. The normal rostrocaudal patterning of the somites (as assayed by the metameric pattern of heparan sulfate proteoglycan in the rostral half-sclerotome and peanut lectin and chondroitin sulfate proteoglycan in the caudal half-sclerotome), combined with our *in vitro* data, suggest that the primary effect of soluble ephrin-B1 is on the neural crest.

Time-lapse cinematography revealed major defects and decreased directionality in the trajectories of the ephrin-B1-Fc-treated neural crest cells (Figure 4; Table 1). Although their overall rates of migration were similar to control and EphB2-Fc-treated explants, neural crest cells moved in a circular and disoriented fashion in the presence of ephrin-B1-Fc, sometimes moving across the intrasomitic border and backwards into the neural tube. This

disorientation is likely to be due to the activation of signal transduction pathways in neural crest cells surrounded by dimeric ephrin-B1-Fc, which may function as a weak agonist. In support of this possibility, preliminary data (not shown) suggest that neural crest cells display normal directionality in the presence of monomeric ephrin-B1-myc, which does not activate signaling [32]. These data, in combination, provide interesting mechanistic information about how Eph receptors might exert their function in neural crest cells, suggesting a potential link between receptors and cytoskeletal elements.

Our previous studies demonstrated that treatment of trunk explants with PNA, which binds to cells in the caudal sclerotome [8,44], resulted in neural crest cells migrating through both rostral and caudal halves of the somite [13]. However, time-lapse recordings reveal different migratory behaviors in the presence of PNA compared with ephrin-B1-Fc. In soluble ephrin-B1-Fc-treated explants, both rostrally and caudally-located neural crest cells traveled in a non-linear, disoriented manner (Figure 4). In PNA-treated explants, by contrast, rostrally located neural crest cells moved normally, while caudally located neural crest cells traveled in a highly disorganized manner [13], resembling neural crest cells in the presence of dimeric ephrin-B1-Fc (Figure 4). This similarity suggests that neural crest cells may be receiving repulsive signals in both cases, either from the exogenous ephrin-B1-Fc or from the ephrin-B1-expressing caudal sclerotome cells. However, induction of an abnormal collapse response cannot be the sole explanation for the disruption of the segmental pattern reported here. Ephrin-B1-myc, which does not stimulate receptor phosphorylation and signaling [32], alters segmental migration without eliciting abnormal cell behaviors. The differential effects observed with PNA and ephrin-B1-Fc treatment demonstrate that there are likely to be multiple inhibitory mechanisms in the caudal sclerotome.

The addition of exogenous receptor EphB2-Fc had no effect on the segmental pattern, rates or continuance of neural crest migration either *in vitro* or *in vivo*. This may result from the highly adaptive nature of Eph family receptor–ligand interactions, as observed in previous studies on the growth of optic axons [25,39]. In stripe assays, optic axons can adapt to high basal levels of ligand and grow normally on concentrations dramatically higher than normally required for growth cone collapse and repulsion. Similarly, the axons become exquisitely sensitive to very low levels of ligand in conditions with no ligand. Thus, if even a fraction of the ligand remained free to interact in the presence of EphB2-Fc, adaptation would result in cells that are more sensitive, generating a near-normal response. Alternatively, the failure of the inhibition may suggest that EphB2-Fc does not abolish a higher-order interaction between receptors and ligands, or an asymmetry in Eph receptor–ligand interactions.

Our functional studies, performed on neural crest cells migrating *in situ*, are in agreement with complementary studies using different assay systems. Both ephrin-A5 and ephrin-A2 act to inhibit axon outgrowth in the retinotectal system [21,25,45]; ephrin-A5 has also been implicated in axon fasciculation by cortical neurons [23]. Ephrin-A5, expressed by skeletal muscle, inhibits axon outgrowth and may regulate topographically appropriate neural connectivity in the peripheral nervous system [46]. Eph receptors and ligands act in earlier events of neuronal patterning: expression of a truncated EphA4 receptor disrupts the normal segmental pattern of *Krox-20* expression in the hindbrain of frogs and zebrafish [29], and EphA4, EphB1 and ligand ephrin-B2 influence the segmental organization of branchial arch neural crest migration [47].

Chick and rat both exhibit metameric expression patterns of EphB receptors and ephrin-B ligands within the developing trunk somites, but different members are expressed in each species. Recently, Wang and Anderson [37] demonstrated in rat that the ligands ephrin-B2 and ephrin-B1 have an inhibitory influence on trunk neural crest cells and motor axons *in vitro*. Rat neural crest cells primarily express EphB2, whereas chick neural crest cells predominantly express EphB3 (Figure 1). In addition, EphB2 and ephrin-B2 are expressed by rat rostral and caudal sclerotome [37], respectively, whereas EphB3 and ephrin-B1 are present in chick rostral and caudal sclerotome (Figures 1,2). In rat, *ephrin-B1* transcripts are expressed by cells at the dorsal lip of the dermomyotome [37] and not by caudal sclerotome cells as observed here in chick and quail (Figure 2). These findings suggest that there may be evolutionary conservation within this Eph subclass, but not necessarily of individual receptors and ligands.

Conclusions

These results demonstrate the presence of Eph family receptor tyrosine kinases on neural crest and rostral half-sclerotomal cells, and their cognate ligands in the caudal half of the sclerotome. Their distribution is consistent with previous proposals that Eph receptors and their ligands play roles in establishing boundaries between regions, probably by mediating repulsive interactions [18]. Furthermore, the presentation of exogenous ephrin-B1 in a uniform, non-graded fashion disrupts the segmental patterning of neural crest migration without necessarily activating signal transduction. By visualizing neural crest migration in real time in the presence of blocking reagents, the present experiments offer direct evidence for Eph ligand–receptor interactions in shaping the migratory routes of neural crest cells.

Materials and methods

Whole embryo staining

EphB2 receptor and ephrin-B1 ligand fusion proteins were constructed as described [32]. Chick and quail embryos were stained as described [18]. Whole-mount embryos were photographed and then prepared for

standard cryostat sectioning. Images were captured using a Roche ProgRes camera on Zeiss Axiophot or Stemi SV11 microscopes and processed with Adobe Photoshop 3.0 software.

cDNA probes and in situ *hybridization*

EphB2 and *EphB3* cDNAs were isolated by screening at high stringency a 4 day quail cDNA library with a PCR fragment of the tyrosine kinase domain of the QEK5 gene [48]. Two cDNAs were isolated: *EphB2* [24] and *EphB3* (encoding proteins with 98% and 91% amino acid similarity, respectively, to chick EphB3 [33] and human EphB3 [49,50]). *In situ* hybridizations were carried out as described [51], except that post-antibody washes were done overnight at 4°C in MABT (maleic acid buffer–tween). The two probes used for *EphB3* were: a 1 kb *Pvu*II fragment spanning approximately 500 bp of the extracellular domain and 500 bp of the intracellular domain; and a 1.8 kb fragment encompassing most of the intracellular domain and about 700 bp of the 3′ untranslated region. The probes used for *EphB2* were: a 1150 bp fragment encompassing 1 kb of the intracellular domain and 150 bp of the 3′ untranslated region; and the entire 3.1 kb *EphB2* cDNA. cDNA probes for *ephrin-B1* and *ephrin-B2* were provided by H. Wang and D. Anderson. Anti-HNK-1 antibody [52] was used to identify neural crest cells in cryosections of embryos that had been processed for *in situ* hybridization.

Neural crest cultures

Stripes of alternating substrates were prepared as described [40,53] with a few modifications. 35 mm UV-sterilized culture dishes were first coated with a nitrocellulose/methanol solution and allowed to dry under a sterile hood, followed by application of a sterile stripe matrix. Stripes were filled three times for 20 min with a solution containing 100 μg/ml fibronectin, polyclonal goat anti-IgG-Fc antibody (250 μg/ml; Jackson Immunoresearch), and Texas Red-BSA (10 μg/ml; Sigma), washed and incubated with 200 μg/ml fibronectin in phosphate-buffered saline (PBS) for 1 h, followed by a solution of ephrin-B1-Fc (10 μg/ml) in PBS for 2 h, followed by two rinses in PBS and addition of neural crest culture medium. For some experiments, instead of adding antibody and ligand-Fc sequentially, a solution of ephrin-B1-Fc (10 μg/ml) and the goat anti-IgG-Fc antibody was preincubated for 1 h at room temperature before this solution was applied as alternating stripes, as described above. A fibronectin solution was then applied over the entire dish. To some dishes, monomeric ephrin-B1-myc (1 or 10 μg/ml) or dimeric ephrin-B1-Fc (1 or 10 μg/ml) was added to the culture medium.

Primary neural crest cell cultures were prepared from quail embryos, at stages 12–14 [33], according to standard procedures [53]. Neural tubes were arranged perpendicular to the striped substrata and placed in a 5% CO_2 culture incubator. Cultures were photographed at 12 and 24 h using fluorescence and phase optics. For time-lapse analysis using a BioRad MRC 600 confocal microscope (LD Acroplan 40× long working distance objective), Hepes (pH7.7; 20 mM final) was added to the medium and cultures were maintained at 37°C in a warming incubator. Images were collected every minute for 40 min to 3 h and were analyzed using NIH Image 1.61 software.

Whole trunk explants

Trunk explants were prepared as described [13,14] after prelabeling the neural tubes with DiI [38]. Trunk regions were pre-incubated in a solution (0.25–10 μg/ml) of sterile-filtered ligand (ephrin-B1-Fc or ephrin-B1-myc), EphB2-Fc or Fc alone in culture medium for 4 h at 37°C. They were placed on Millicell inserts (Millipore) and grown at 37°C in a 5% CO_2 tissue culture incubator for 24–36 h. Some explants were fixed overnight in 4% paraformaldehyde after 24–36 h in culture and stained with anti-HNK-1 antibody to mark neural crest. HNK-1-labelled neural crest cells were counted in the rostral and caudal portions of somites in ephrin-B1-Fc-treated explants, ephrin-B1-myc-treated explants and control Fc-treated explants using a Zeiss Axiovert equipped with fluorescence and phase optics. Statview software was used to determine the mean ± standard deviation, and to perform a Student's two-tailed *t*-test.

Time-lapse videomicroscopy of whole trunk explants

DiI-labeled neural crest cells were followed by low-light-level videomicroscopy as previously described [13]. Time-lapse frames were converted to digital images using the MetaMorph software (Universal Imaging Corporation, West Chester, PA). The digital images were transferred to a Silicon Graphics workstation. Individual cells were followed at 30 min intervals and their rates of migration were analyzed using a cell tracking program (J. Solomon and S. Speicher, Computational Biology Center, Beckman Institute, California Institute of Technology; unpublished data).

Receptor phosphorylation assays

Receptor phosphorylation was determined as described [18,32].

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